

**METHOD FOR DETECTING MICROORGANISMS**

The present invention relates to a method for detecting the elements constituting microorganism flora, at least  
5 some of the elements of which have an operon in common, characterized in that the elements of said flora are identified by studying the intergenic sequence of said operon.

10 The human digestive system harbors a considerable number of microorganisms which constitute a microbial flora of extreme complexity. Although these bacteria are distributed throughout the digestive tract, the  
15 colon contains most of the flora, both from a quantitative and a qualitative point of view. It is estimated that the colonic flora of an individual consists of  $10^{13}$  to  $10^{15}$  bacteria, mostly anaerobic bacteria, represented by at least 400 species belonging to approximately 30 different genera. These bacteria  
20 colonize the various stages of the colon in a relatively heterogeneous manner. A "fermentation flora" in the cecum and also a "putrefaction flora" in the left colon are conventionally described. Moreover, a residence flora is distinguished from a passing flora.  
25 This residence flora is itself divided up into dominant flora and subdominant flora. The dominant bacteria, essentially anaerobic bacteria, are mainly represented by the *Bacteroides* genus, gram-negative bacilli, but also by the *Bifidobacterium*, *Lactobacillus* or  
30 *Clostridium* genera, gram-positive bacilli. The subdominant flora contain aero-anaerobic and microaerophilic bacteria. The presence of enterobacteria and of streptococci is especially noted. Diet, infections, intake of pre- and probiotics and  
35 also treatments with antibiotics are all liable to cause drastic modifications in the composition of the colonic flora. Since these variations have a direct impact on health, it is important to be aware of them

and to understand them, both in order to avoid them and in order to trigger them for therapeutic purposes. To date, the study of colonic bacteria has made it possible to characterize approximately 200 species.

5 However, this type of investigation comes up against many problems essentially associated with the laboriousness of the techniques. The results obtained and also the conclusions which ensue therefrom are still sketchy.

10

Bacterial identification is carried out according to various methods which employ either the demonstration of specific phenotypical or biochemical characteristics, or the use and recognition of specific  
15 heterologous regions present on the genome.

The beginnings of an identification may be carried out by describing the morphology of the organism studied and by searching, for example, for the presence of  
20 endospores, of sheaths, of cysts, of buds, of fruiting bodies, etc. The form of the colonies, the pigmentation, and the origin of the sample are also useful pieces of information. Preliminary studies may also be carried out using dyes specific for the  
25 capsule, the flagella, the granules, the wall, etc. However, a more thorough and precise identification necessarily involves techniques comprising isolation on selected media. Such media are developed or improved in order to increase the specificity of the selection.  
30 However, it is difficult to completely exclude the presence of possible contaminants which may then interfere in the recognition tests subsequently employed.

35 These tests make use of the biochemical characteristics specific for a species. Multitest systems exist. They are identification galleries which are provided in the form of kits, of microplates and of strips, the use of which is sometimes automated. However, there exists a

certain degree of heterogeneity of chromosomal or plasmid origin within many species. Thus, one or more characteristics will be absent in the identification. Thus, most of the time, only the probability of  
5 belonging to a species will be given. A similarity of 80% or more with a reference bacterium will be considered as acceptable. Single-test systems have also been developed. They use synthetic fluorescent substrates for revealing the presence of an enzyme  
10 specific for a microorganism. They enable a rapid analysis, but are limited in their use. Specifically, a specific test must be developed for each species.

Immunoassays using poly- or monoclonal antibodies have  
15 also been developed. Besides the obvious limitation inherent in polyclonal antibodies, these assays are mainly used to characterize serotypes, but are rarely used to identify species. Although commonly used in hospital diagnosis, they remain relatively unused in  
20 bacteriology. As regards the production of monoclonal antibodies, it remains long, laborious and expensive work. They are, for example, directed against lipopolysaccharides, the membrane, the pili, etc., but are, however, rarely specific for a species.

25 The development of molecular biology techniques has made it possible to develop novel identification assays. These techniques are based on hybridization reactions or polymerase chain amplification reactions  
30 (PCR).

Genome/genome hybridization should be greater than or equal to 70% to identify an unknown bacterial species as being the known reference bacterium whose genomic  
35 DNA is used to perform the diagnosis. Other assays involve heterologous regions on the DNA specific for a species. The hybridization technique can thus be used, which consists in depositing the product to be analyzed on a nylon or nitrocellulose membrane and then in

incubating with a specific labeled probe (cold probe or hot probe) {1}.

5 It is also possible to use specific primers making it possible to amplify a fragment of a given size by the PCR technique {2, 3}. In this case, the amplicates obtained by PCR can themselves be analyzed by other techniques, such as RFLP (restriction fragment length polymorphism) {4} or TGGE (temperature gradient gel electrophoresis) {5}, refining the diagnosis.

15 Despite their large capacity for discrimination, these techniques remain limited since they make it possible to analyze only one species at a time, or else consist in isolating a mixture of species which cannot then be identified without knowing exactly the pattern of analysis of the amplicates by a given technique on a given biotope.

20 The development of DNA chips (biochips) makes it possible to envision a rapid diagnosis relating to several hundreds of species. This technique consists in placing on a surface area of a few millimeters squared several hundreds of DNA sequences specific for a given organism. These probes are hybridized with DNA fragments, generally obtained by RT-PCR. The possible hybridization of said fragments is then observed, and indicates the presence or absence of the gene expressed, or of the organism studied.

30 The nucleic acid targets studied over the last few years are essentially 16S ribosomal RNA (with more than 7000 available sequences) {1, 2, 4, 5}, the region separating the 16S and 23S genetic loci {3} and the elongation factors {6, 7}. Thus, using 16S RNAs as a basis, it has been possible to detect several new species, belonging to the *Bacteroides* and *Clostridium* groups, but not to the *Bifidobacterium* group {8}. Moreover, and although 16S RNAs make it possible to

detect and to identify many bacteria down to the species, they are incapable of discriminating between the various species of staphylococci {2, 3}. Thus, a variable region of the gene encoding HSP 60 has been  
5 proposed for studying the microorganisms of the intestinal flora {9}.

A subject of the present invention is a method for detecting and identifying the elements constituting a  
10 microorganism flora, in particular the intestinal flora, according to which a target which is even more discriminatory and universal than those already studied is detected and studied.

15 The method according to the present invention comprises characterizing the sequences of this target for the organisms present in the microbial flora studied, and thus makes it possible to design a diagnostic test.

20 Thus, the target studied in the method of the invention exhibits strong interspecies heterogeneity, which allows discrimination between the microorganisms.

The present invention therefore relates to a method for  
25 detecting the elements constituting a microorganism flora, at least some of the elements of which have an operon in common, characterized in that:

30 a) the genomic DNA of said flora or the mRNAs is (are) prepared,

b) at least some of the noncoding intergenic sequences located in the operon conserved in at least some of the elements of the flora are  
35 amplified, and

c) the various intergenic sequences amplified are identified in order to determine the elements of said flora.

Specifically, surprisingly, it has been noted that the intergenic regions, in the operons conserved between various species, exhibit a certain heterogeneity, whereas the coding regions which flank said regions in the 5' and/or in the 3' position are generally very conserved. It is possible that this fact is due to a relatively weak selection pressure on the noncoding regions in the course of evolution {10}.

The amplification is preferably carried out by polymerase chain reaction (PCR), but other methods (PCR-like) may be employed, using a pair of primers having nucleotide sequences for implementing the method according to the invention.

The term "PCR-like" is intended to denote all methods using direct or indirect reproductions of nucleic acid sequences, or else in which the labeling systems have been amplified; these techniques are, of course, known. In general, it involves amplification of the DNA with a polymerase; when the sample of origin is an RNA, a reverse transcription should be carried out beforehand. A very large number of methods currently exist for this amplification, such as, for example, the SDA (Strand Displacement Amplification) technique, the TAS (Transcription-based Amplification System) technique, the 3SR (Self-Sustained Sequence Replication) technique, the NASBA (Nucleic Acid Sequence Based Amplification) technique, the TMA (Transcription Mediated Amplification) technique, the LCR (Ligase Chain Reaction) technique, the RCR (Repair Chain Reaction) technique, the CPR (Cycling Probe Reaction) technique, or the Q-beta-replicase amplification technique. Some of these techniques have since been improved.

The analysis of the amplified sequences is advantageously carried out on a DNA kit comprising

sequences complementary to the sequences liable to be amplified from the elements of said flora. Knowledge of the microorganisms which may be present in the biological sample studied is therefore important in order to choose the DNA chip to be analyzed. Thus, it is necessary for the DNA chip to have, at its surface, probes specific for each of the organisms intended to be studied. Such a DNA chip is also a subject of the invention.

Thus, the present invention relates most particularly to a DNA chip comprising, at its surface, a plurality of oligonucleotides complementary to the intergenic sequences of the various operons conserved between the species. The term "DNA chip" is intended to mean a solid support to which are attached nucleic acid fragments under conditions which allow hybridization thereof with the complementary oligonucleotides, and detection of the hybrids thus formed. Thus, a DNA chip according to the invention also relates to the membranes as used to perform Southern blotting.

The oligonucleotides attached to the DNA chip according to the present invention are so attached by any conventional method known to those skilled in the art, and are approximately 50 bases long. It is understood that the oligonucleotides considered may also be shorter or longer. Thus, it is within the scope of those skilled in the art to determine the length of the oligonucleotides attached to the chip according to the invention, for each sequence.

Preferably, the oligonucleotides attached to the DNA chip are chosen such that their sequence comprises a part of the hypervariable region identified according to the present invention. The oligonucleotides attached to the chip according to the invention may also contain sequences corresponding to the sequences variable to a

lesser degree, located at or close to the end of the operon genes.

In a particular and preferred implementation of the invention, the DNA chip according to the invention has a plurality (a number greater than or equal to 2, preferably 3, more preferably 5, most preferably 10) of oligonucleotides greater than 40 bases long. Preferably, said oligonucleotides comprise a fragment of at least 20, preferably 40 or 50, more preferably 75, most preferably 100 consecutive bases, of the sequences SEQ ID No. 63 to SEQ ID No. 138 and SEQ ID No. 140 to SEQ ID No. 189, corresponding to the intergenic sequences of various species (rpoBC for SEQ ID No. 63 to SEQ ID No. 138, GroESL for SEQ ID No. 140 to SEQ ID No. 189).

Thus, demonstrating the possible hybridizations of the amplified sequences makes it possible to identify the elements present in the microbial flora studied.

An operon which is particularly suitable for implementing the method according to the invention is the bacterial rpoBC operon. This bacterial operon contains coding sequences which are relatively homologous between genera. It is therefore possible to determine degenerate primers for amplifying a region which is heterologous between species and which corresponds to the transcribed intergenic region (IGR). In bacteria, the rpoBC operon encodes the beta and beta prime subunits of DNA-directed RNA polymerase, just like the homologous genes, which may or may not be conserved in the form of an operon, in mitochondria and other eukaryotic organelles (chloroplasts), and just like nuclear eukaryotic RNA polymerase II (which synthesizes the messenger RNAs). The study of this operon makes it possible not only to detect the bacteria, but also other eukaryotic microorganisms (yeast, protozoa, or others).



The method according to the invention is thus carried out using degenerate primers located in the coding sequences of the operons, in particular at least one primer chosen from the sequences SEQ ID No. 1 to SEQ ID No. 31, themselves a subject of the invention. The RNA polymerase proteins are in fact extremely conserved according to the species, which makes it possible to find amino acid sequences which align with one another, and thus to choose degenerate oligonucleotides for amplifying the intergenic sequences.

The pairs of primers described by the sequences: (a sequence chosen from the sequences SEQ ID No. 1 to SEQ ID No. 8)/(a sequence chosen from the sequences SEQ ID No. 9 to SEQ ID No. 11) are used to perform a first amplification of the intergenic, IGR, of the bacteria. A second, more specific, amplification can then be carried out using pairs of primers which hybridize within the first amplified region, and which are described by the sequences: (a sequence chosen from the sequences SEQ ID No. 12 to SEQ ID No. 15)/(a sequence chosen from the sequence SEQ ID No. 16 to SEQ ID No. 31).

25

SEQ ID N°1	GGN GAY AAR YTN GCN GGN AGN CAY GG
SEQ ID N°2	GGN GAY AAR YTN GCN GGN CGN CAY GG
SEQ ID N°3	GGN GAY AAR YTN GCN AAY AGN CAY GG
SEQ ID N°4	GGN GAY AAR YTN GCN AAY CGN CAY GG
SEQ ID N°5	GGN GAY AAR ATG GCN GGN MGN CAY GG
SEQ ID N°6	GGN GAY AAR TTY GCN TCN MGN CAY GG
SEQ ID N°7	GGN GAY AAR TTY GCN AGY MGN CAY GG
SEQ ID N°8	GGN GAY AAR TTY GCN ACN MGN CAY GG
SEQ ID N°9	AAY GCN GAY TTY GAY GGN GAY CAR AT
SEQ ID N°10	AAY GCN GAY TTY GAY GGN CAR ATG GC
SEQ ID N°11	AAY GCN GAY TTY GAY GGN GAY GAR AT
SEQ ID N°12	GGN GGN CAR MGN TTY GGN GAR ATG GA
SEQ ID N°13	GGN GGN CAY GGN TTY GGN GAR ATG GA
SEQ ID N°14	GGN GGN CAR WSN TTY GGN GAR ATG GA
SEQ ID N°15	GGN GGN NTN MGN TTY GGN GAR ATG GA
SEQ ID N°16	GGN AAR CGN GTN GAY TAY TCN GGN MG
SEQ ID N°17	GGN AAR CGN GTN GAY TAY AGN GGN MG

SEQ ID N°18	GGN AAR AGN GTN GAY TAY TCN GGN MG
SEQ ID N°19	GGN AAR AGN GTN GAY TAY AGN GGN MG
SEQ ID N°20	GGN AAR CGN GGN GAY TAY TCN GTN MG
SEQ ID N°21	GGN AAR CGN GGN GAY TAY AGN GTN MG
SEQ ID N°22	GGN AAR AGN GGN GAY TAY TCN GTN MG
SEQ ID N°23	GGN AAR AGN GGN GAY TAY AGN GTN MG
SEQ ID N°24	GGN AAR CGN GTN GAY TTY TCN GGN MG
SEQ ID N°25	GGN AAR CGN GTN GAY TTY AGN GGN MG
SEQ ID N°26	GGN AAR AGN GTN GAY TTY TCN GGN MG
SEQ ID N°27	GGN AAR AGN GTN GAY TTY AGN GGN MG
SEQ ID N°28	GGN AAR CGN GTN GAY TTY TCN GCN MG
SEQ ID N°29	GGN AAR CGN GTN GAY TTY AGN GCN MG
SEQ ID N°30	GGN AAR AGN GTN GAY TTY TCN GCN MG
SEQ ID N°31	GGN AAR AGN GTN GAY TTY AGN GCN MG

The pairs of primers described by the sequences: (a sequence chosen from the sequence SEQ ID No. 53 to SEQ ID No. 54 are used to amplify a the intergenic region, IGR, of the bacteria.

FO	SEQ ID N°53	GGN GGN CAN NSN TTY GGN GAR ATG GA
RP	SEQ ID N°54	AAY GCN GAY TTY GAY GGN GAY SAR AT
FO	SEQ ID N°55	GGN GGN CAR MGN TTY GGN GAR ATG GA
	SEQ ID N°56	GGN GGN CAY GGN TTY GGN GAR ATG GA
	SEQ ID N°57	GGN GGN CAR WSN TTY GGN GAR ATG GA
	SEQ ID N°58	GGN GGN NTN MGN TTY GGN GAR ATG GA
RP	SEQ ID N°59	AAY GCN GAY TTY GAY GGN GAY CAR AT
	SEQ ID N°60	AAY GCN GAY TTY GAY GGN CAR ATG GC
	SEQ ID N°61	AAY GCN GAY TTY GAY GGN GAY GAR AT

These primers were designed based on the study of the degeneracy of conserved protein motifs corresponding to rpoB and/or encoded by the rpoB gene:

beta 2 I :  
 coryneb/bif/actinom/camp/pseudom/salmon/esch/vibrio  
 /clos/bact/hel/citrob/prot/haf/yers/  
 /past/actinob/aer  
 SEQ ID N°55 GGN GGN CAR MGN TTY GGN GAR ATG GA  
 (8 deg)  
 beta 2 ii : bacillus  
 SEQ ID N°56 GGN GGN CAY GGN TTY GGN GAR ATG GA  
 (7 deg)  
 beta 2 iii : helicobacter mustelae  
 SEQ ID N°57 GGN GGN CAR WSN TTY GGN GAR ATG GA  
 (8 deg)  
 beta 2 iv : archae (methano)  
 SEQ ID N°58 GGN GGN NTN MGN TTY GGN GAR ATG GA  
 (9 deg)  
 FO : 2 I/II/III : GGN GGN CAN NSN TTY GGN GAR ATG GA  
 (SEQ ID N° 53)

5 For the reverse sequences, determined based on the  
 degeneracy of conserved protein motifs corresponding to  
 rpoC and/or encoded by the rpoC gene

beta p 2 i :  
 coryneb/bif/actinom/bac/camp/pseudom/salmon/esch/vi  
 brio/clos/bact/hel/citrob/prot  
 /haf/yers/past/actinob/aer/staph/lactob/enteroc/lactoc  
 SEQ ID N° 59 AAY GCN GAY TTY GAY GGN GAY CAR AT (8  
 deg)  
 beta p 2 ii : archae (methano)  
 SEQ ID N° 61 AAY GCN GAY TTY GAY GGN GAY GAR AT (8  
 deg)  
 beta p 2 iii : streptoc  
 SEQ ID N° 60 AAY GCN GAY TTY GAY GGN CAR ATG GC (7  
 deg)  
 RP : P 2 i/ii : AAY GCN GAY TTY GAY GGN GAY SAR AT  
 (SEQ ID N° 54)  
 « REVERSE » ATY TSR TCN CCR TCR AAR TCN GCR TT  
 (SEQ ID N° 62)

10 These primers are also part of the invention.

A subject of the invention is also the genomic  
 sequences of microorganisms which may be amplified by  
 the primers according to the invention, in particular

the pairs of primers: (a sequence chosen from the sequences SEQ ID No. 1 to SEQ ID No.8)/(a sequence chosen from sequences SEQ ID No. 9 to SEQ ID No. 11), and the pairs of primers: (a sequence chosen from the sequences SEQ ID No. 12 to SEQ ID No. 15)/(a sequence chosen from the sequences SEQ ID No. 16 to SEQ ID No. 31). Amplification with pairs of primers: (a sequence chosen from the sequences SEQ ID No. 53, SEQ ID No. 55 to SEQ ID No. 58)/(a sequence chosen from the sequences SEQ ID No. 54, SEQ ID No. 59 to SEQ ID No. 61) is also envisioned.

Thus, a subject of the invention is also in particular a sequence from SEQ ID No. 63 to SEQ ID No. 138, which correspond to the hypervariable intergenic regions of the rpoB operon or various organisms. A subject of the invention is also a fragment of a minimum of 20 bases, preferably 30 bases, more preferably 50 bases, even more preferably 75 bases, most preferably 100 bases of one of the sequences SEQ ID No. 63 to SEQ ID No. 138, or the sequences complementary thereto, it being possible for said fragment to be used to define organism-specific primers, or for the identification of organisms, in particular by hybridization.

Thus, the DNA chip according to the invention preferably has, at its surface, a plurality of oligonucleotides (a minimum of two) comprising fragments chosen from the fragments of the sequences SEQ ID No. 63 to SEQ ID No. 138 defined above, thus allowing the identification of microorganisms. The length of these oligonucleotides can be determined by those skilled in the art, as a function of the hybridization conditions which they intend to use. Oligonucleotides approximately 50 bases long are thus envisioned.

Another operon which is particularly suitable for implementing the method according to the invention is

the bacterial GroESL operon. This bacterial operon is bicistronic and contains coding sequences which are relatively homologous between genera. It is therefore also possible to determine degenerate primers to  
5 amplify a region which is heterologous between species and which corresponds to the transcribed intergenic region (IGR). In bacteria, the GroESL operon encodes the HSP10 and HSP60 proteins (heat shock proteins of 10 and 60 kDa respectively), just like the homologous  
10 genes, which may or may not be conserved in the form of an operon, in mitochondria and other eukaryotic organelles (chloroplasts). The study of this operon makes it possible not only to detect bacteria, but also other eukaryotic microorganisms (yeasts, protozoa, or  
15 others).

The method according to the invention is thus carried out using degenerate primers located in the coding sequences of the operons. The HSP proteins are in fact  
20 extremely conserved according to species, which makes it possible to find amino acid sequences which align with one another, and thus to choose degenerate oligonucleotides to amplify the intergenic, promoter or terminator sequences.

25 Preferably, the primers described by the sequences SEQ ID No. 32 and SEQ ID No. 33 are used to amplify the intergenic region, IGR, of *E. coli* and of Enterobacteriaceae.

30

ENT-BDEG:

CTGGAYGTGAARRTNGGYGAYATYGT

(SEQ ID N° 32)

ENT-ADEG:

ANNACNGTNGCRGTRGTGGTIRCCGTC

(SEQ ID N° 33)

Other degenerate primers can also be used to implement the protocol according to the invention, in particular

any primer chosen from the sequences SEQ ID No. 34 to  
SEQ ID No. 52.

UNI-ADEG 1:	
GGNGAYGGNACNACNACNGCNACNNT	(SEQ ID N° 34)
UNI-ADEG 2:	
GGNGAYGGNACNACNACNTGNTCNNT	(SEQ ID N° 35)
ENT-BNEW:	
AANMTTCGTCCNYTRCANGAYCGNGT	(SEQ ID N° 36)
CLO-BNEW2:	
ATNARRCCAYTWGGWGAYMGNGTWGT	(SEQ ID N° 37)
BIF-BNEW:	
AARCCRCTCGAGGACMRNRTNSTSGT	(SEQ ID N° 38)
UNI-A3:	
GGNGAYGGNACNAANACNGCNACNNT	(SEQ ID N° 39)
BIF-BNEW2:	
ATCAAGCCNCTMGRRGACMRSRTNST	(SEQ ID N° 40)
HEL-BNEW:	
NTNCANCCNTTNGGNGANAGNGTNTT	(SEQ ID N° 41)
CAM-BNEW:	
NTNCANCCNTTNGGNAANCGNGTNCT	(SEQ ID N° 42)
BACT-BNEW:	
NTNAANCCNTTNGCNGANCGNGTNCT	(SEQ ID N° 43)
CHLA-BNEW:	
NTNAANCCNTTNGGNGANAGNATNTT	(SEQ ID N° 44)
MYCP-BNEW:	

NTNAAACCNNTNGGNAANCGNGTNAT STA-BNEW:	(SEQ ID N° 45)
NTNAAACCNNTNGGNAANCGNGTNAT LACC-BNEW:	(SEQ ID N° 46)
TTGAAACCNNTTAGNGRAYCGYGTRST LACB-BNEW:	(SEQ ID N° 47)
TTAMARCCAWTMGGNGATCGNGTNRT CLO-BNEW3:	(SEQ ID N° 48)
ATNANACCANTNGGNGACAGNGTNGT ENT-BNEW2:	(SEQ ID N° 49)
NTNCGNCCNNTTNCANGANCGNGTNAT LEG-BNEW:	(SEQ ID N° 50)
NTNCGNCCNNTTNCANGANCGNGTNGT AER-BNEW:	(SEQ ID N° 51)
NTNCGNCCNCTNCANGANCGNGTNAT LACB-BNEW2:	(SEQ ID N° 52)
MARCCNNTNGGNGAYMGNGTNATNGT	(SEQ ID N° 139)

5 These primers are also subjects of the present invention. Preferably, the detection of a microorganism is carried out using a pair of primers SEQ ID No. 32/ SEQ ID No. 33, or (SEQ ID No. 34, SEQ ID No. 35 or SEQ ID No. 39)/(a sequence chosen from the sequences SEQ ID No. 36 to SEQ ID No. 38 or SEQ ID No. 40 to SEQ ID No. 52).

10 The sequences SEQ ID No. 36 to SEQ ID No. 38 and/or SEQ ID No. 40 to SEQ ID No. 52 and/or SEQ ID No. 139, used in particular in amplification reactions with sequences SEQ ID No. 34, SEQ ID No. 35 and/or SEQ ID No. 39, make  
15 it possible, respectively, to detect the microorganisms and species listed below. One or more pair(s) of sequences may be used in an amplification reaction.

20 Thus, the sequences according to the present invention make it possible in particular to detect microorganisms of the following genera and families: Lactococcus (SEQ ID No. 39), Bifidibacterium (SEQ ID No. 38 and/or 40),

Mycobacterium (SEQ ID No. 40), Helicobacter (SEQ ID No. 41), Campylobacter (SEQ ID No. 42), Bacteroides (SEQ ID No. 43), Chlamydia (SEQ ID No. 44), Mycoplasma (SEQ ID No. 45), Staphylococcus (SEQ ID No. 46), Lactococcus  
5 and/or Streptococcus (SEQ ID No. 47), Lactobacillus and/or Bacillus (SEQ ID No. 48), Clostridium (SEQ ID No. 37 and/or 49), Enterobacteriaceae (SEQ ID No. 36 and/or 50), Pasteurella and/or Haemophilus (SEQ ID No. 50), Neisseria and/or Legionella (SEQ ID No. 51),  
10 Aeromonas and/or Bordetella (SEQ ID No. 52), Lactobacillus and/or Bacillus (SEQ ID No. 139).

The subject of the invention is also the genomic sequences of microorganisms which can be amplified  
15 using the primers according to the invention, in particular the pairs of primers SEQ ID No. 32/ SEQ ID No. 33, and (SEQ ID No. 34, SEQ ID No. 35 or SEQ ID No. 39)/(a sequence chosen from the sequences SEQ ID No. 36 to SEQ ID No. 38, SEQ ID No. 40 to SEQ ID No. 52 or SEQ  
20 ID No. 139).

Thus, a subject of the invention is also in particular a sequence from SEQ ID No. 140 to SEQ ID No. 189, which correspond to the hypervariable intergenic regions of  
25 the GroESL operon of various organisms. A subject of the invention is also any fragment of a minimum of 20 bases, preferably 30 bases, more preferably 50 bases, even more preferably 75 bases, most preferably 100 bases of one of the sequences SEQ ID No. 140 to SEQ ID  
30 No. 189, or the sequences complementary thereto, it being possible for said fragment to be used to define organism-specific primers, or for the identification of organisms, in particular by hybridization.

35 Thus, the DNA chip according to the invention preferably has, at its surface, a plurality of oligonucleotides (a minimum of two) comprising fragments chosen from the fragments of the sequences SEQ ID No. 140 to SEQ ID No. 189 defined above, thus



allowing the identification of the microorganisms. The length of these oligonucleotides can be determined by those skilled in the art, as a function of the hybridization conditions, which they intend to use.  
5 Oligonucleotides approximately 50 bases long are thus envisioned.

Subjects of the present invention are also diagnostic kits for carrying out the method according to the  
10 invention. These diagnostic kits contain degenerate primers for amplifying one or more intergenic regions of operon which is conserved among species. They may also contain the reagents required for the amplification reaction. Moreover, DNA representing  
15 positive or negative controls may be included in the diagnostic kits according to the invention.

A diagnostic kit according to the invention also advantageously contains the elements required for  
20 analyzing the amplified products. In particular, a diagnostic kit according to the invention contains a DNA chip according to the invention, which has, at its surface, the sequences corresponding to the various microorganisms.

25 Depending on the species it is desired to detect, the diagnostic kit according to the invention contains the appropriate pair of primers and analytical elements. Furthermore, a kit according to the invention may also  
30 comprise instructions for carrying out the method according to the invention.

A diagnostic kit according to the invention may also only contain a DNA chip according to the invention, and  
35 optionally also instructions for carrying out the analysis of fragments located in the intergenic region of operons which are conserved between species, the preferred operons being GroESL and rpoBC.

The coupling of specific primers and probes thus allows rapid and precise identification of the flora of a given individual. It is therefore possible to establish the profile(s) of populations characteristic of healthy individuals. It is also possible to establish the standard profiles for various pathological conditions.

The method according to the invention also provides the possibility of easy monitoring of the evolution of the flora as a function of diet. More specifically, it is possible to follow the effects, in the colon, of a particular food, such as a pre- or probiotic, or of a medicinal treatment such as a treatment with antibiotics.

It is therefore possible to envision the development of foods or medicinal products for "effect on the flora" purposes, allowing reestablishment and a return to a normal profile after an imbalance subsequent to any pathological condition or attack. It is also possible to use primers and probes corresponding to pathogenic strains in order to optionally establish critical population thresholds preceding a pathological condition. It is then possible to determine which of the other populations are liable to exert a barrier effect on these pathogens.

The tools for diagnosing the intestinal flora developed and based on the method according to the invention (which are also subjects of the invention) are of interest firstly to industrial companies in the agrofoods and pharmacy domains, in order to develop their products and to determine the impact thereof on the intestinal flora. Specifically, particular diets are liable, in the long term, to significantly modify the composition of the flora and, consequently, to have harmful or beneficial effects depending on the types of population which appear or disappear. Similarly, medicinal treatments, and in particular antibiotic

treatments, lead to imbalances in the microflora. Characterization of the populations affected according to the type of medicinal product would make it possible to set up a parallel or subsequent treatment capable of preventing these modifications or of reestablishing a correct flora as rapidly as possible.

These tools are also of interest to health professionals, for characterizing the intestinal flora of patients, which may make it possible, for example, to direct a treatment. Specifically, gastroenterologists estimate that 70% of the population of industrialized countries complain of diverse digestive disorders, which are called functional colopathy, ranging from simple digestive disorders such as bloating or flatulence, to more significant disorders such as constipation or diarrhea, etc. The majority of their consultations concern this functional colopathy.

Few solutions are provided to treat these disorders since, for certain types of colopathy, their cause is still quite unknown, and for others, there is no effective treatment. Added to this is the problem of the medical diagnosis since the patients presenting these symptoms of functional colopathy do not generally present any physical lesion in the colon. Only a questionnaire enables the gastroenterologist to turn toward a type of treatment, which proves to be relatively ineffective in the majority of cases. The market for products which can relieve these disorders is therefore considerable, as is that for diagnosis. Specifically, a diagnosis of the state of the flora of the patients might provide the physician with information regarding the causes of their disorders and the treatment to be carried out.

In order to select a genomic target of interest, namely a target which is conserved in all the genomes, the conservation of the most conserved operons in the

course of evolution was studied based on the genomes of the 51 bacteria entirely sequenced and available on the NCBI server. These sequences were positioned relative to that of rpoB/C (beta operon).

5

It emerged from this first analysis that the longest and most conserved targets are in fact the GroESL operons (encoding Hsp10 (groES) and Hsp60 (groEL)) and a part of the beta operon corresponding to the rpoB and  
10 rpoC genes (encoding the beta and beta' subunits of DNA-directed RNA polymerase). In addition, it was possible to identify conserved protein motifs sufficiently long to allow the definition and then the synthesis of universal (ubiquitous), or almost  
15 universal, degenerate primers.

Thus, these two operons were chosen in order to exemplify the principle of the method according to the invention.

20

Finally, the region of interest of the beta operon was amplified, i.e. the region amplifiable by PCR, using the two corresponding degenerate primers (FO and RP: SEQ ID No. 53 and SEQ ID No. 54) for selection of  
25 bacteria in order to establish the sequence thereof and to test them by hybridization on a nylon membrane so as to validate this specificity. These sequences were also aligned to their homologs available on GenBank in order to observe this specificity by bioinformatics.

30

The same experiments were carried with the GroESL operon, and it can thus be shown that the method according to the invention makes it possible to identify and discriminate between the various species  
35 of microorganisms.

The following examples are intended to illustrate the invention, and should not be considered as limiting the invention.

In the application, the abbreviations for the bacteria are as follows:

- Bacillus subtilis (BS) CIP 52-65T; Bacteroides vulgatus (BV) DSM 1447; Bifidobacterium longum (BL) DSM 20219;  
5 Clostridium leptum (CL) DSM 753; Clostridium nexile (CN) DSM 1787; Clostridium spiroforme (CS) DSM 1552; Clostridium glycolycum (CG) DSM 1288; Lactobacillus gasei (LG) DSM 20077; Lactobacillus helveticus (LH) CIP 103146; Lactobacillus paracasei (LP) DSM 8741;  
10 Lactobacillus reuteri (LR) DSM 20053; Pseudomonas aeruginosa (PA) CIP100720; Ruminococcus hydrogenotrophicus (RH) DSM 10507; Citrobacter freundii (CF); Serratia liquefaciens (SL); Serratia marcescens (SM); Enterobacter cloacae (EnC); Escherichia coli  
15 (EsC); Morganella morganii (MM); Proteus mirabilis (PM); Klebsiella oxytoca (KO); Klebsiella pneumoniae (KP).

#### DESCRIPTION OF THE FIGURES

- 20 Figure 1: Diagram of the rpoBC operon of *E. coli*. The universal (ubiquitous) primers are used to amplify the intergenic sequence.

- Figure 2: Diagram of the groESL operon of *E. coli*. The universal primers are used to amplify the intergenic  
25 sequence.

Figure 3: Principle of a DNA chip. Specific sequences are attached to a solid support. The possible hybridization of the complementary sequences makes it possible to determine their presence in a sample.

- 30 Figure 4: Hybridization of deposits of 10 ng of DNA amplified by PCR with rpoBC primers (i) and of genomic DNA (ii), with a *Serratia marcescens* probe (~ 0.25 ng/ml) (A) or a *Klebsiella oxytoca* probe (~ 1 ng/ml) (B) for 18 hours at 60°C and revelation for  
35 30 minutes at 37°C. It is possible to observe cross hybridization of CF, SM, SL, EC and KP with the KO-DIG probe (~ 1 ng/ml) and of SL with the SM-DIG probe (~ 0.25 ng/ml).

Figure 5: Hybridization of deposits i (genomic DNA, a: 10 to 20  $\mu$ g, b: 5 to 10  $\mu$ g, c: 0.5 to 1  $\mu$ g, d: 50 to 100 ng, e: 5 to 10 ng, f: 0.5 to 1 ng) and ii (DNA amplified by PCR with GroESL primers. a: 50 to 100 ng, b: 5 to 10 ng, c: 0.5 to 1 ng, d: 50 to 100 pg, e: 5 to 10 pg, f: 0.5 to 1 pg) with a PA-DIG probe (~ 10 ng/ml) for 18 hours at 42°C and revelation for 30 min at 37°C.

Figure 6: Hybridization of deposits i (DNA amplified by PCR with rpoBC primers: 10 ng/1 ng/100 pg) and ii (genome DNA: 1  $\mu$ g/100 ng/10 ng) with an LR-DIG probe (~ 1 ng/ml) for 18 hours at 50, 55, 60 and 65°C and revelation for 30 min at 37°C.

#### EXAMPLES

##### Example 1: Isolation of strains

In order to have a broad and representative sample of human colonic flora, it is necessary to isolate new bacterial strains, also called nonculturable (and therefore unknown) strains, which constitute a high percentage of the bacteria of the human colonic flora.

In order to perform these isolations, a large quantity of human stools is collected and sterilized by means of gamma-type radiation or by heat, for the purpose of sealing therein samples of these same human stools and of culturing them aerobically and anaerobically, in liquid and solid media.

Depending on the culture conditions used, it is thus possible to isolate new genera, species or strains of colonic bacteria or other eukaryotic microorganisms.

##### Example 2: Characterization of the sequences of the isolated strains (rpoB)

This involves carrying out the molecular characterization of sequences, ideally of mRNA to

perform a quantification, and if not of genomic DNA, of the isolates of bacterial or eukaryotic microorganisms.

5 The sequences corresponding to portions of the bacterial rpoBC operon are studied. The genes of this operon are in fact relatively homologous between genera.

10 A computer analysis (sequence alignment) thus makes it possible to define degenerate primers for amplifying the region which is heterologous between species and which corresponds to the transcribed intergenic region.

15 Thus, the primers SEQ ID No. 1 and SEQ ID No. 31, and also the other primers, were defined after alignment of the sequences corresponding to more than 50 species of living organisms (prokaryotes and eukaryotes, not shown) using the redundancy of the genetic code. The sequences SEQ ID No. 53 and SEQ ID No. 54 are in particular  
20 preferred.

The regions amplified by PCR or RT-PCR with the abovementioned primers can obviously be cloned into various vectors, in order to be used to refine the  
25 analysis (in particular in order to sequence them).

Example 3: Characterization of the sequences of the isolated strains (GroESL)

30 The sequences corresponding to portions of the bicistronic bacterial GroESL operon are studied. The genes of this operon are in fact relatively homologous between genera.

35 Computer analysis (sequence alignment) thus makes it possible to define degenerate primers for amplifying the region which is heterologous between species and which corresponds to the transcribed intergenic region.

Thus, the primers SEQ ID No. 34 and SEQ ID No. 35 were defined after alignment of the sequences corresponding to more than 100 species of living organisms (prokaryotes and eukaryotes, not shown).

5

The sequences SEQ ID No. 36 to SEQ ID No. 52, and in particular SEQ ID No. 139, correspond to complementary sequences which can be used to amplify microorganisms of diverse genera and/or families.

10

As regards the primers SEQ ID No. 32 and SEQ ID No. 33, they were defined based on the conserved sequences of the GroES and GroEL genes of *E. coli*, using the degenerative genetic code.

15

Example 4: Amplification reactions (GroESL)

The PCR reactions are carried out according to the following protocol:

20

2 ml of culture broth shaken at 37°C for 18 h are concentrated by centrifugation and resuspension of the bacterial pellet in 30 µl of distilled water, and then a 1/10 dilution of this concentrate, treated at 100°C for 10 minutes, is used as a matrix for the PCR reactions. The reaction conditions are 94°C/5 min, then 25 cycles of (94°C/30 sec, 60°C/45 sec, 72°C/30 sec), followed by an elongation step at 72°C for 7 min.

25

30 Analysis of the amplificates makes it possible to show that it is possible to amplify, using the primers SEQ ID No. 32 and SEQ ID No. 33, the intergenic region of various enterobacteria, such as *Escherichia coli*, *Enterobacter cloacae*, *Morganella morganii*, *Serratia*  
35 *liquefasciens*, *Proteus mirabilis*, *Serratia marcescens*, *Klebsiella pneumoniae*, *Citrobacter freundii* or *Klebsiella oxytoca*. The amplified region varies in length, according to the species, from 400 to 500 base pairs (bp). Use of the pair SEQ ID No. 34 and SEQ ID



No. 36 gives amplicates of between 550 and 650 bp in length.

5 Use of the pairs: (SEQ ID No. 34, SEQ ID No. 35 or SEQ ID No. 39)/(a sequence chosen from the sequences SEQ ID No. 36 to SEQ ID No. 38 or SEQ ID No. 40 to SEQ ID No. 52, or SEQ ID No. 139) makes it possible to amplify sequences specific to certain families and species, and to identify the organisms of these families or species.

10

For the amplification reactions, use is preferably made of a primer marked "A" with a primer marked "B".

15 The regions amplified by PCR or RT-PCR with the abovementioned primers can obviously be cloned into various vectors, in order to be used to refine the analysis (in particular in order to sequence them).

#### PCR PROTOCOL

20

In order to show that using the intergenic region of the two operons of interest as a nucleic acid probe can make it possible to discriminate several bacterial species, said IGR for each target was amplified by direct PCR on bacterial suspensions. For the amplification reactions, use is preferably made of a primer marked "A" with a primer marked "B".

25 2 ml of culture broth shaken at 37°C for 18 h are concentrated by centrifugation and resuspension of the bacterial pellet in 30 µl of distilled water, and then a 1/10 dilution of this concentrate, treated at 100°C for 10 minutes, is used as a matrix for the PCR reactions.

35

#### groESL OPERON:

The PCR reactions for this target are carried out at a  $T_m$  ranging between 59°C and 60°C. The reaction

conditions are 94°C/5 min, then 25 cycles of (94°C/30 sec, 60°C/45 sec, 72°C/30 sec), followed by an elongation step at 72°C for 7 min. The amplified intergenic regions are then observed by agarose gel electrophoresis using a 1 Kb + ladder (Gibco BRL).

Analysis of the amplificates makes it possible to show that it is possible to amplify, using the primers SEQ ID No. 32 and SEQ ID No. 33, the intergenic region of various Enterobacteria, such as *Escherichia coli*, *Enterobacter cloacae*, *Morganella morganii*, *Serratia liquefaciens*, *Proteus mirabilis*, *Serratia marcescens*, *Klebsiella pneumoniae*, *Citrobacter freundii* or *Klebsiella oxytoca*. The amplified region varies in length, according to species, from 400 to 500 base pairs (bp). Use of the pair SEQ ID No. 34 and SEQ ID No. 36 gives amplificates of between 550 and 650 bp in length.

#### 20 rpoB/C OPERON:

The PCR reactions for this target are carried out at a  $T_m$  ranging between 63°C and 64°C. The reaction conditions are 94°C/4 min, then 30 cycles of (94°C/30 sec, 64°C/30 sec, 72°C/3 min), followed by an elongation step at 72°C for 12 min. The amplified intergenic regions are then observed by agarose gel electrophoresis using a molecular weight marker III DNA ladder (ref: No. 528552; Boehringer Mannheim).

Analysis of the amplificates makes it possible to show that it is possible to amplify, using the pair of primers SEQ ID No. 53 and SEQ ID No. 54, the intergenic region of the various bacteria, such as *Escherichia coli*, *Clostridium leptum*, *Klebsellia oxytoca*, *Lactococcus lactis*, *Citrobacter freundii*, *Serratia marcescens*, *Proteus mirabilis*, *Serratia liquefaciens*, *Morganella morganii*, *Enterobacter cloacae* or *Ruminococcus hydrogenotrophicus*.

DNA fragments corresponding to the intergenic regions of the rpoB/C operon in various species were reamplified and analyzed using bands extracted from an agarose gel preparation. These fragments were prepurified with a Qiagen extraction kit.

The regions amplified by PCR or RT-PCR with the abovementioned primers can obviously be cloned into various vectors, in order to be used to refine the analysis (in particular in order to sequence them).

#### HYBRIDIZATION PROTOCOL

With a view to testing the specificity of the PCR products, for the species selected for our study, deposits of these DNAs were made on a nylon membrane according to a sodium hydroxide (NaOH) fixation protocol. The DNA concentrations for these deposits are given on the corresponding figures. These membranes were hybridized according to the protocol of the PCR DIG Probe Synthesis Kit (Roche) Cat. No. 1636090. The concentration of the probe used, synthesized according to the same protocol, is also indicated on the figures, as is the temperature of hybridization carried out overnight (18 h). The temperature of pre-hybridization is 65°C for each experiment, and it lasts 45 min.

Detection of this type of hybridization with this type of labeling (DIG) is termed colorimetric ("cold" labeling different from radioactive labeling).

Figures 4 to 6 show a specificity detection as a function of the organisms, although some crosshybridization reactions may exist. These reactions may be reduced by choosing probes which are shorter and located among the hypervariable intergenic sequences, as defined by SEQ ID No. 63 to SEQ ID No. 138 (rpoN) or SEQ ID No. 40 to SEQ ID No. 189 (GroESL).

Thus, a DNA chip with various probes located in the intergenic region will make it possible to recognize without hesitation the presence or absence of a  
5 microorganism, even when there is crosshybridization. Specifically, the presence of a microorganism will be deduced from the hybridization for each of the probes.

10 It may therefore be advantageous to define DNA chips having specific probes corresponding to the intergenic region of each microorganism, but also to include several different probes for each microorganism.

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